




PATENT
Docket No. 432722002623

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Marian Christopher

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Gregory R. MUNDY, et al.

Serial No.: 09/695,807

Filing Date: October 23, 2000

For: INHIBITORS OF PROTEASOMAL
ACTIVITY FOR STIMULATING BONE
GROWTH

Examiner: R. Gitomer

Group Art Unit: 1651

**DECLARATION OF I. ROSS GARRETT
PURSUANT TO 37 C.F.R. § 1.132**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, I. Ross Garrett, declare as follows:

1. I am one of the co-inventors of the above-referenced patent application, and am familiar with the contents thereof.

2. Other co-inventors and I have conducted experiments demonstrating that proteasome inhibitors promote osteoblast proliferation, differentiation of osteoblast precursors and bone growth. These experimental results are set forth in the following paragraphs 3-10 and in the attached Tables 1-6 and Figures 1-2.

3. Osteoblast proliferation, differentiation of osteoblast precursors and bone growth can be shown by histologic observations of calvarial bone cultures, which show increased osteoblast numbers, BMP induction, new bone formation and morphologic maturation of osteoblasts. Detailed disclosure of methods for determining new bone area and osteoblast numbers is in the specification at page 20, line 11 to page 21, line 11 and in Example 2 at page 35, line 16 through page 36, line 26.

4. Compounds that stimulate bone growth can be shown by analyzing the induction of BMP mRNA and protein. Disclosure of these methods for identification of bone anabolic agents is found in the specification at page 3, lines 24-25, page 19, lines 7-11, and page 20, lines 5-6. Analysis of mRNA and protein expression can be performed using assays well-known in the art.

5. Osteoblast proliferation and bone growth can be shown by histomorphometric analysis of bone following *in vivo* treatment with a compound of interest. Detailed disclosure of methods for morphometric analysis is found in the specification at page 21, line 12 to page 24, line 9, Examples 3 and 4 at page 37 to page 39, and Example 7, at page 41.

6. Promotion of osteoblast proliferation and new bone growth by structurally-unrelated proteasome inhibitors is demonstrated in the Table 1 submitted herewith. A number of these compounds were tested for their capacity to stimulate bone formation in calvarial organ cultures and inhibit proteasomal activity assay as described in Paragraph 3 *supra*. The ten structurally unrelated proteasomal inhibitors examined included epoxomicin, AcLL, eponemycin, lactacystin, YU101, AcNaphLLL, PSI, MG132, MG115, and ALLN. Data are expressed as ED₅₀, the dose required to elicit half of the maximal response for either bone formation or proteasomal inhibitory activity. The ability of the compounds to inhibit proteasomal activity was determined using proteasome activity as described in specification at Example 5. The ability to stimulate bone formation was determined using the *in vitro* bone formation assay exemplified in Example 2. Specifically, explants of neonatal murine calvarial bone were cultured for 72 hours in the presence of the compound and then examined histomorphometrically. A strong positive correlation ($R^2=0.94$) was demonstrated between the capacity of each of these compounds to inhibit proteasomal activity and their bone forming activity. The most potent and selective inhibitor of the chymotrypsin-like site, the amino-

terminally acetylated epoxyketone peptide YU101, stimulated bone formation at concentrations of 10 nM.

7. The specificity of proteasome inhibitors on BMP-2 expression was determined by examining the effects of the proteasome inhibitors PSI on mRNA expression of BMP-2, BMP-4, BMP-6 in fetal calvarial cells (FRC) osteoblasts *in vitro* as described in Paragraph 4 *supra*. The proteasomal inhibitors PSI increased BMP-2 mRNA expression, but had no significant effects on mRNA expression of BMP-4 and BMP-6 as shown in Figure 1.

8. Proteasomal inhibitors were examined for their capacity to increase accumulation of BMP2 protein in conditioned media from human Hu09 osteoblastic cells by coculturing the cells with the inhibitors at multiple concentrations for 24 hours as shown in Table 2. Protein levels in the media were determined using a commercially available ELISA kit for BMP2 (Quantikine, R&D Systems). The structurally-unrelated proteasomal inhibitors tested were YU101, proteasome inhibitor-1, epoxomicin, MG132, MG115, and lactacystin. Data are expressed as mean \pm SEM where * designates $p < 0.05$ versus vehicle alone. All of the proteasomal inhibitors tested induced statistically significant increases in the BMP-2 protein production from osteoblastic cells when compared to cells treated with vehicle alone.

9. The inhibitory ability of structurally-unrelated proteasomal inhibitors positively correlated with the ability to stimulate the expression of BMP-2 protein in Hu09 osteoblastic cells as shown in Table 3. The proteasomal inhibitors epoxomicin, lactacystin, YU101, PSI, MG132, and MG115 were analyzed as described in Paragraph 4 *supra*. Data are expressed as the doses expressed as ED₅₀ are the doses required to elicit half of the maximal response for BMP2 protein production and proteasomal activity inhibition. In every case, the ability to inhibit proteasomal activity correlated with the induction of BMP-2 expression ($R^2=0.95$).

10. The ability of structurally-unrelated proteasomal inhibitors to stimulate bone formation positively correlated with the ability to stimulate the expression of BMP-2 protein in Hu09 osteoblastic cells as shown in Table 4. The proteasomal inhibitors epoxomicin, lactacystin, YU101, PSI, MG132, and MG115 were analyzed as described in Paragraphs 3 and 4 *supra*. Data are expressed as the doses expressed as ED₅₀ are the doses required to elicit half of the maximal response for either BMP2 protein production and bone formation. In every case, the ability to stimulate bone formation correlated with the induction of BMP-2 expression ($R^2=0.95$).

12. The ability of proteasome inhibitors to stimulate new periosteal bone formation in the calvaria of mice was determined as described in Paragraph 5 *supra*. To perform these experiments, compounds were injected into the subcutaneous tissue over the calvaria of normal mice. 5-weeks old Swiss ICR white mice were injected 3 times/day for 5 days with either vehicle alone, epoxomicin or PSI over the right side of the calvarium. Mice were euthanized on day 22 and calvaria removed for histomorphometric analysis. Data are expressed as mean +/- SEM where * designates $p < 0.001$ versus treatment with vehicle alone. Both PSI and epoxomicin stimulated new bone formation in a dose-dependent manner and did not cause toxic effects in this dose range as shown in Table 5.


13. *In vivo* administration of proteasomal inhibitors also demonstrated the ability of these inhibitors to stimulate bone formation using the methods as described in Paragraph 5 *supra*. Briefly, histologic sections, bone formation rates and mineral apposition rates of murine proximal tibia were determined from mice treated daily for 5 days with (a) vehicle alone, (b) PTH at 0.08 mg/kg/day subcutaneously, (c) PSI at 2 mg/kg/day intraperitoneally, (d) epoxomicin at 0.1 mg/kg/day intraperitoneally. The mice were subsequently sacrificed 16 days later for bone analysis. In Figure 2, values in parentheses are percent change from vehicle-treated controls. BV/TV bone volume/tissue volume; BFR, bone formation rate; MAR mineral apposition rate. PSI was administered subcutaneously and epoxomicin was administered intraperitoneally to normal intact Swiss white mice for 5 days. Parathyroid hormone (PTH) was used as a control, since when used over longer periods it has powerful anabolic effects on bone formation. However, PTH did not have significant effects with only 5 days of daily subcutaneous administration (Figure 2b). PSI caused an increase in trabecular bone volume of 28 percent accompanied by a 71 percent increase in bone formation rates measured by dynamic parameters compared with vehicle-treated mice (Figure 2c). This increase in bone formation rate was present when the animals were sacrificed, which was over two weeks after the compound was last administered. This increased bone formation rate shows the increase in trabecular bone volume was due to an absolute increase in the rate of bone formation rather than a secondary effect due to a decreased bone resorption. There were no effects of these compounds on rates of bone resorption in organ cultures and the measured increases in rates of bone formation are so pronounced that the increases in bone volume cannot be ascribed to a simple reduction in bone

turnover. Epoxomicin (Figure 2d) caused similar responses to those of PSI, but at doses at least 20-fold less.

14. The effects of proteasome inhibitors on trabecular bone volume in mice was determined as described in Paragraph 5 *supra*. PSI was injected subcutaneously, and epoxomicin was injected intraperitoneally in normal intact Swiss white mice. PSI was injected into mice at 1-2 mg/kg/day for 5 days. Epoxomicin was injected into mice at doses between 0.5-0.004 mg/kg/day for 5 days. The data shown in Table 6 demonstrate that treatment with epoxomicin and PSI caused a significant increase in bone formation in a dose responsive manner. Data were expressed as mean +/- SEM where * designates $p < 0.001$ versus treatment with vehicle alone. At the highest dose of epoxomicin, a 98% increase in the bone volume/tissue volume. The increases in bone formation rates and bone volumes after only 5 days of treatment are of comparable degree to those that require over one month of treatment with other anabolic agents such as the statins and PTH.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

4-22-2003
Date



I. Ross Garrett

TABLE 1

<u>Compound</u>	<u>Bone Formation Activity (nM)</u> ED ₅₀	<u>Proteasome Inhibitory Activity (nM)</u> ED ₅₀
Epoxomicin	10	100
AcLLL	3000	3000
Eponemycin	250	625
Lactacystin	2000	1000
YU101	10	60
AcNaphLLL	40	200
PSI	32	100
MG132	500	625
MG115	1000	1000
ALLN	5000	1600

TABLE 2

Treatment	Concentration (uM)	BMP2 protein (pg/ml)
YU101	Vehicle alone	37 ± 5
	0.0025	56 ± 15
	0.005	59 ± 20
	0.010	119 ± 22*
	0.020	163 ± 12*
	0.040	212 ± 27*
Proteasome inhibitor-1	Vehicle alone	67.2 ± 5
	0.0062	98 ± 12*
	0.0125	139 ± 15*
	0.025	158 ± 18*
	0.050	152 ± 22*
	0.100	156 ± 16*
Epoxomicin	Vehicle alone	36 ± 6
	0.01	50 ± 8
	0.02	108 ± 14*
	0.04	172 ± 9*
	0.08	75 ± 11*
MG132	Vehicle alone	71 ± 13
	0.035	65 ± 4
	0.075	84 ± 13
	0.100	107 ± 11*
	0.300	140 ± 14*
	0.600	145 ± 6*
MG115	Vehicle alone	71 ± 12
	0.300	71 ± 11
	0.600	149 ± 15*
	0.1200	74 ± 3
Lactacystin	Vehicle alone	36 ± 6
	125	37 ± 8
	250	83 ± 12*
	500	117 ± 15*

TABLE 3

Compound	Proteasome Inhibitory Activity (nM) ED ₅₀	BMP2 expression (nM) ED ₅₀
Epoxomicin	100	10
Lactacystin	1000	300
YU101	60	15
PSI	100	20
MG132	625	150
MG115	1000	300

TABLE 4

Compound	Bone Formation Activity (nM) ED ₅₀	BMP2 expression (nM) ED ₅₀
Epoxomicin	10	10
Lactacystin	2000	300
YU101	10	15
PSI	32	20
MG132	500	150
MG115	1000	300

TABLE 5

Treatment	Dose (mg/kg/day)	Total Bone Area (um ²)	% Increase
PS1	Vehicle alone	0.64 ± 0.03	
	0.1	0.74 ± 0.02*	22
	1	0.83 ± 0.02*	35
	5	0.79 ± 0.03*	32
Epoxomicin	Vehicle alone	0.68± 0.02	
	0.05	0.78±.03*	15
	0.1	0.87±.02*	28
	0.5	0.91±.03*	34

TABLE 6

Treatment	Dose (mg/kg/day)	% BV/TV	% Increase
PS1	Vehicle alone	13.1 ± 2.2	
	1	22.3 ± 2.4*	36
	2	17.6 ± 1.2*	25
Epoxomicin	Vehicle alone	11.7 ± 3.4	
	0.004	14.3 ± 1.8	22
	0.02	18.5 ± 1.0*	58
	0.1	23.2 ± 4.2*	98

FIGURE 1




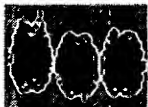


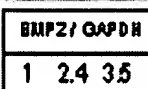
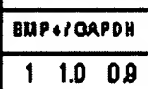
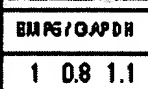




0	50	100	0	50	100	0	50	100	PS1 (nM)
									BMPs
									
									GAPDH
									BMPs
BMP2 / GAPDH			BMP4 / GAPDH			BMP6 / GAPDH			
1 2.4 3.5			1 1.0 0.9			1 0.8 1.1			

FIGURE 2

				
BV/TV	13.8 ± 1.60	16.1 ± 1.20 (+17)	17.6 ± 1.40 (+28)*	19.6 ± 4.90 (+42)*
BFR	0.14 ± 0.02	0.17 ± 0.07 (+21)	0.24 ± 0.02 (+71)*	0.33 ± 0.03 (+136)*
MAR	1.04 ± 0.07	1.06 ± 0.08 (+2)	1.16 ± 0.09 (+12)	1.36 ± 0.10 (+31)*